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A Fluorescence Polarization Competition Immunoassay for Tyrosine Kinases

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We have recently reported a homogeneous, nonradioactive fluorescence polarization method to assay protein tyrosine kinase activity. Our original approach can only be used with a peptide substrate and requires large amounts of anti-phosphotyrosine antibody. To overcome these problems an alternate fluorescence polarization competition immunoassay was designed and evaluated. In this assay, phosphorylated peptide or protein produced by kinase reaction will compete with a fluorescent phosphopeptide used as a tracer for immunocomplex formation with phosphotyrosine antibody. In this format kinase activity will result in the loss of the polarization signal. To validate the fluorescence polarization competition immunoassay, Lck activity was compared with a more commonly used $^{32}PO_4$-transfer assay using Lck peptide or enolase as the substrate. In both the assays, Lck activity showed a similar dependence on ATP, Lck enzyme, and the peptide/enolase substrate c ncentrations with the FP signal inversely proporti nal to the amount of $^{32}PO_4$ transferred to the substrate. Inhibition by staurosporine and the Lck inhibitor 4-amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine was similar in these two assays. The advantages f this assay over other kinase assays include use of nonisotopic substrates and a more simple procedure in which the kinase assay is done in a single tube (well of a microtiter plate), without separation, precipitation, or washing. This method is easily automated for high-throughput drug discovery screening. © 1998 Academic Press

Protein tyrosine kinases (PTKs)[2] are a diverse group of protein kinases that include transmembrane receptor PTKs (several growth factor receptors) and nonreceptor cytoplasmic PTKs. Src-family PTKs play important roles in the regulation of growth, proliferation, and differentiation (1–3). Several of these PTKs are drug discovery targets. Existing assay procedures are not readily automated, use radioactive substrate, and/or are tedious. Hence, it is desirable to develop a simple, reliable PTK assay to screen compounds for effectors as a first step in developing drugs for a variety of diseases.

Several PTK assays have been used to assay protein kinase activity. These include $^{32}PO_4$-transfer assay (solid phase and liquid phase) (4, 5), ELISA (6), and DELFIA (fluorometric) (7) assay. The radioactive assays use radioactive [γ-^{32}P]- or [^{33}P]ATP as PO_4 donor and some problems include half-life, storage, usage, and disposal of the large amounts of radioactive waste generated. Substrate-coated plates are used in ELISA, DELFIA, and solid-phase radioactive assay, and the coating step is time consuming (8–10). In addition, the plates are coated with scintillant in solid-phase radioactive assay (Flash plate) making these assays very expensive. Furthermore, these assays include several wash, liquid transfer, and incubation steps and hence are labor intensive and time consuming.

We recently described a nonradioactive, homogeneous, FP-Lck assay (11). In this FP-Lck assay the product formed, fluorescein-phosphopeptide, is immunocomplexed to anti-PY antibody. The small fluorescent peptide substrate, which has free rotational mobility, is converted to a larger fluorescent phosphopeptide–antibody complex with restricted rotational mobility. Substrate (unphosphorylated peptide) will not bind antibody, and an increase in polarization allows one to monitor the reaction's progress.

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[2] Abbreviations used: PTK, protein tyrosine kinase; Lck, lymphoid T-cell protein tyrosine kinase; FP, fluorescence polarization; PY, phosphotyrosine; GST, glutathione S-transferase; Mops, 3-[*N*-morpholino]propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; DELFIA, dissociation-enhanced lanthanide fluorescence immunoassay; milli-P, millipolarization units; PP, 4-amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; DMSO, dimethyl sulfoxide.

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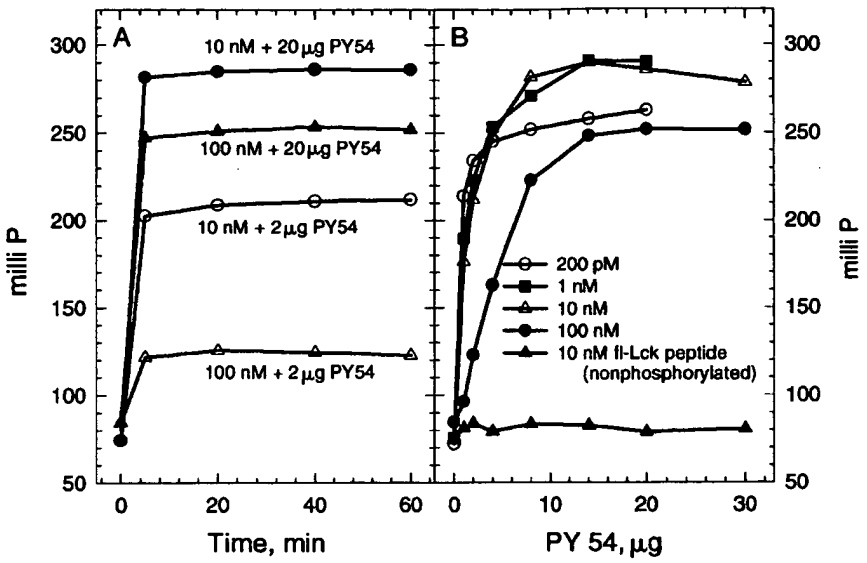

FIG. 1. Association of fluorescein-labeled tyrosine-phosphorylated Lck-peptide (fl-phos-peptide) with phosphotyrosine antibody PY 54. (A) Time course of immunocomplex formation with fl-phos-peptide and PY 54. To 10 and 100 nM of fl-phos-peptide, 2 or 20 μg of PY 54 was added and incubated for 5, 20, 40, and 60 min and FP signal was measured. The binding of fl-phos-peptide to PY 54 was rapid and stable and reached a maximum within 5 min. (B) Titration of fl-phos-peptide with PY 54. To different concentrations of fl-phos-peptide, PY 54 was added in 1 ml of buffer (20 mM Mops containing 20 mM EDTA), incubated at room temperature in the dark for 30 min, and read in the FPM-1.

Although a significant improvement as an automatable procedure, our original FP-kinase assay has some drawbacks. Only peptide substrates can be used, kinetic analysis of the peptide substrate dependence is complex, and large amounts of antibody are required (11). To overcome these difficulties, we have designed and evaluated a FP-competition immunoassay. In this method the kinase phosphorylates a nonfluorescent peptide or protein and the phosphorylated peptide or protein formed in the reaction will compete with fluorescent tyrosine-phosphorylated peptide used as a tracer that forms an immunocomplex with anti-phosphotyrosine antibody as in a more typical immunoassay.

MATERIALS AND METHODS

Materials. The PTK used in the FP assay was a recombinant mouse Lck fused to GST, expressed in Sf 9 baculovirus, purified on glutathione resin, concentrated to 1 mg/ml, and stored at −80°C. Lck is a 56-kDa nonreceptor protein kinase of the Src-family and is essential for the development of T-cells and activation of T-cells by antigen. Lck-peptide substrate ala-glu-glu-glu-ile-tyr-gly-val-leu-phe-ala-lys-lys-lys-lys and fluorescent tyrosine-phosphorylated peptide (fl-phos-peptide) fluorescein-ala-glu-glu-glu-ile-tyr(P)-gly-val-leu-phe-ala-lys-lys-lys-lys were synthesized by Research Genetics. Monoclonal anti-phosphotyrosine (PY) antibody PY 54 was obtained from Transduction Labs. Ascites (ascitic fluid) of anti-phosphotyrosine antibody was raised by injecting 5H1 hybridoma cells into mice. Ascites was comparable with PY 54 in affinity to bind to fl-phos-peptide. [γ-^{32}P]ATP was from NEN-Dupont. Rabbit muscle enolase and all other fine chemicals used were of the highest purity and purchased from Sigma Chemical Co. The sources of all other reagents are as described in Ref. 11.

Fluorescence polarization-competition immunoassay. In the FP-Lck assay peptide substrate is incubated with Lck (0.5 μg/ml) and 0.1 mM ATP in the assay buffer (50 mM Mops, 15 mM $MgCl_2$, and 5 mM $MnCl_2$, pH 7) in a final volume of 100 μl in a 12 × 70-mm borosilicate glass tube at 25°C. After incubation the reaction is terminated with 100 μl EDTA (100 mM) containing 5–20 nM fl-phospho-peptide. PY 54 phosphotyrosine antibody (1–3 μg) or 0.5–1 μl of 5H1 ascites is added in 800 μl of 20 mM Mops, pH 7, containing 20 mM EDTA. Following a 30-min incubation, the FP signal was measured in the FPM-1 fluorescence polarization analyzer (Jolley Consulting and Research Inc.). In the FPM-1 monochromatic light passes through an excitation filter (485 nm) and a moving polarizer and excites fluorescent molecules in the sample. The polarized light in the sample is emitted at right angles to the excited light and then passes through an emission filter (535 nm) and a vertical fixed polarizer and proceeds to a photomultiplier tube. The emitted light is measured in both horizontal (I_h) and vertical (I_v) planes and polarization (P) is calculated from the equation $P = I_v - I_h/I_v + I_h$. FPM-1 measures the vertical and horizontal fluorescence intensity and calculates the total intensity ($I_v + 2I_h$) of the emitted light and polarization in millipolarization units. Fluo-

rescein concentration is quantitated from the total intensity measurements. When inhibitors were used, 10 mM stock solutions were made in DMSO, diluted fresh in the assay buffer, and added to the reaction. In the inhibition assay, the final DMSO concentration does not exceed 1% and DMSO controls are employed.

For validation of the FP-Lck-competition immunoassay, Lck activity by FP was compared with the enzyme activity assayed with the $^{32}PO_4$-transfer assay from [γ-^{32}P]ATP as described previously (11). The data shown for FP and $^{32}PO_4$-transfer assays are representative of a typical experiment that was repeated two to three times. The error margin within duplicates was less than 4%. The FP and $^{32}PO_4$-transfer assays were run concurrently under similar conditions. The profiles of FP signal and phosphate incorporation were reproducible between experiments.

RESULTS AND DISCUSSION

Interaction of fluorescein-labeled tyrosine-phosphorylated Lck-peptide with anti-phosphotyrosine antibody. The PTK used in developing the FP assay was a recombinant GST-fused mouse Lck. Optimal peptide substrate sequences recognized by different PTKs based on the phosphorylation sites of natural substrates or by using an oriented peptide library were described (12, 13). Lck-peptide substrate ala-glu-glu-glu-ile-tyr-gly-val-leu-phe-ala-lys-lys-lys-lys was synthesized and phosphorylation of this substrate by Lck was measured with [^{32}P]ATP in a P-81 filter assay. The Lck-peptide substrate was phosphorylated with a K_m of 16.6 μM as determined by Lineweaver–Burk plot (data not shown) which agrees with published values (14).

As reported earlier (11) the affinity of fl-phos-peptide was highest with PY 54 antibody among different monoclonal and polyclonal anti-phosphotyrosine antibodies tested (data not shown). Titration of different concentrations of fl-phos-peptide with PY 54 and the time course of the fl-phos-peptide and PY 54 interaction are shown in Fig. 1. The fl-phos-peptide binding to PY 54 is rapid and reached equilibrium in less than 5 min and remained unchanged for at least 60 min (Fig. 1A). Different concentrations of fl-phos-peptide (200 pM and 2, 10, and 100 nM) in 1 ml were titrated with increasing amounts of PY 54 antibody (Fig. 1B). At 200 pM fl-phos-peptide the FP signal reached a plateau at 1 μg of PY 54 and at 2 and 10 nM the FP signal increased with increasing amounts of PY 54 and plateaued at 8 μg of PY 54. With 100 nM fl-phos-peptide, 15 μg of PY 54 was required for saturation. These results show that the amounts of antibody needed to obtain the highest FP signal depended on the fl-phos-peptide concentration as expected and reported earlier (11). The dissociation of PY antibody–fl-phos-peptide complex as a function of time was studied (Fig. 2) by competing with different concentrations of phosphorylated unlabeled Lck-peptide. The dissociation was rapid and complete dissociation was achieved within 5 min as shown by the rapid decrease of FP signal in the first 5 min and then remained unchanged. The dissociation increased with increasing concentration of phosphopeptide. These results suggest that the 30-min incubation following the addition of PY antibody in the FP-PTK assay is adequate for competition of the PY antibody–fl-phos-peptide complex.

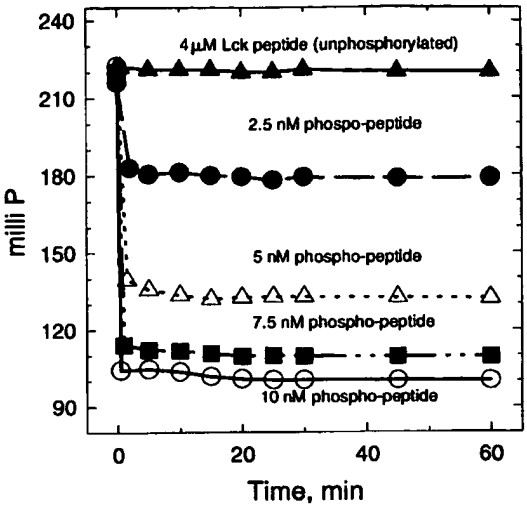

FIG. 2. Dissociation of PY antibody–fl-phos-peptide complex by phosphorylated Lck-peptide as a function of time. Phosphorylated Lck-peptide was made by incubation of 40 μM Lck-peptide (unlabeled with fluorescein) with 200 ng Lck and 200 μM ATP for 2 h and terminating the reaction with the addition of EDTA to 20 mM final concentration. A parallel reaction was run with [γ-^{32}P]ATP to determine the amount of phosphorylated peptide. The PY antibody (2 μg)–fl-phos-peptide (2 nM) complex was formed by a 30-min incubation as measured by the increase of FP signal from 70 to 220–230 milli-P. To the PY antibody–fl-phos-peptide complex, the phosphorylated Lck-peptide (unlabeled) was added and the FP signal was measured as a function of time up to 60 min. The concentration of the phosphorylated Lck-peptide used was indicated on the curves.

In the FP-competition immunoassay, the kinase reaction was performed by incubation of the peptide (or protein) substrate with ATP and Lck enzyme followed by termination of the reaction with EDTA plus fl-phos-peptide, and then antibody was added and the FP signal was measured. The phosphorylated product formed in the assay will compete with the fl-phos-peptide for binding to phosphotyrosine antibody. Kinase activity results in loss of the FP signal and the FP signal is inversely proportional to the phosphorylated product formed in the reaction.

Evaluation of FP-competition immunoassay as a function of ATP, Lck, and Lck-peptide concentration. To show that the FP-competition immunoassay procedure gives results comparable with a more standard kinase protocol, the FP assay and $^{32}PO_4$-transfer reactions were run concurrently. When Lck-peptide (20 μM) phosphorylation was measured as a function of Lck concentration at 0.1 mM ATP, the $^{32}PO_4$ incorpo-

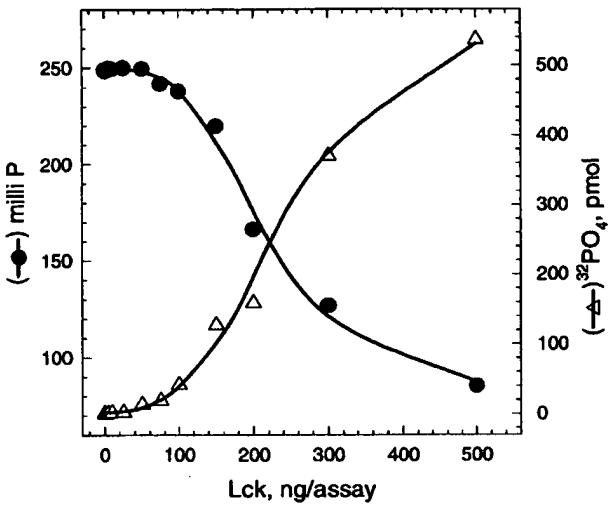

FIG. 3. Dependence of Lck activity on Lck enzyme. Lck activity was measured at 20 μM Lck-peptide, 100 μM ATP, and increasing concentrations of Lck. After the reaction, 5 nM fl-phos-peptide followed by 2 μg of PY 54 was added. The phosphorylated Lck-peptide formed in the reaction competes with fl-phos-peptide and hence the FP signal decreases. There is an inverse correlation between the $^{32}PO_4$ incorporated and FP signal. Each experiment is performed two to three times in duplicate. Each data point is an average of duplicate determinations of a representative individual experiment. The FP and $^{32}PO_4$-transfer assays were done always in parallel. The results are reproducible and the error between duplicates is less than 4%. The profiles of FP signal and $^{32}PO_4$ incorporated were very similar between experiments, though at times different amounts of tracer or antibody were used. The solid-line curves represent the data analyzed by the Logistic.fit curve-fit program of Sigma Plot 2.01.

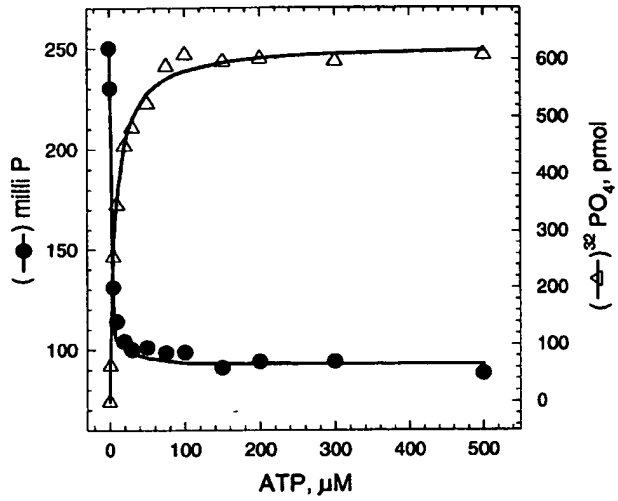

FIG. 4. Lck activity as a function of ATP. Lck activity was measured at 20 μM Lck-peptide and 100 ng Lck at different ATP concentrations. The FP signal rapidly decreased as the $^{32}PO_4$ incorporation increased with increasing ATP concentrations. The solid-line curves were fitted by Hyperbol.fit of Sigma Plot.

rated into Lck-peptide increased initially slowly up to 100 ng and then rapidly up to 500 ng Lck. The FP signal decreased from 250 milli-P slowly up to 100 ng Lck and later more steeply with increasing Lck concentrations and reached 80 milli-P at 500 ng Lck (Fig. 3). The FP signal and $^{32}PO_4$ incorporated were inversely related. Lck activities measured by the FP-competition immunoassay and $^{32}PO_4$-transfer assay agreed very closely. The optimal concentration of 0.5 μg/ml Lck was used in the subsequent assays.

To determine the dependence of the Lck assay on ATP, the assay was performed varying the ATP concentration at 0.5 μg/ml Lck and 20 μM Lck-peptide. When Lck activity was measured by $^{32}PO_4$-transfer assay, $^{32}PO_4$ incorporated into Lck-peptide increased sharply and reached saturation at 50 μM (Fig. 4). The FP signal, as expected, decreased sharply with increasing ATP concentration and was inversely related to $^{32}PO_4$ incorporated. The K_m for ATP were 2.0 and 3.0 μM in the FP-competition immunoassay and $^{32}PO_4$-transfer assay, respectively. Once again the profiles of the FP signal and $^{32}PO_4$ incorporation were inversely related, showing that the FP-Lck-competition immunoassay and the $^{32}PO_4$-transfer assay gave comparable results.

The dependence of the FP signal on peptide concentration was assayed in 100 μl with 100 μM ATP, 0.5 μg Lck/ml, and increasing concentrations of Lck-peptide (Fig. 5). $^{32}PO_4$ incorporated into Lck-peptide increased with increasing peptide concentration and reached saturation at 40 μM. The FP signal started decreasing with increasing concentration of Lck-peptide from 280 to 115 milli-P at 16 μM substrate. The FP signal decreased slowly at higher Lck-peptide concentrations and reached 80 milli-P which is consistent with all the fl-phosphopeptide being competed by the phosphorylated product formed in the reaction. The FP signal and the $^{32}PO_4$ transfer were inversely related.

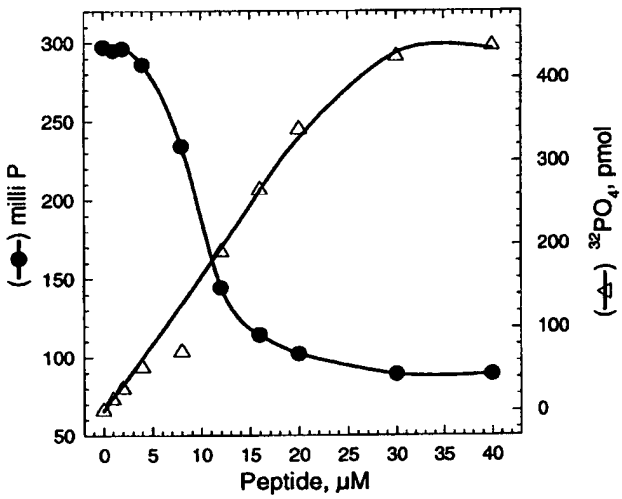

FIG. 5. Dependence of Lck activity on Lck-peptide was assayed at 100 μM ATP and 100 ng Lck. The FP signal decreased with increasing peptide concentration, while the $^{32}PO_4$ incorporated into the peptide increased. The solid line of the FP signal curve is fitted with Logistic.fit and $^{32}PO_4$-transfer curve with Hyperbol.fit.

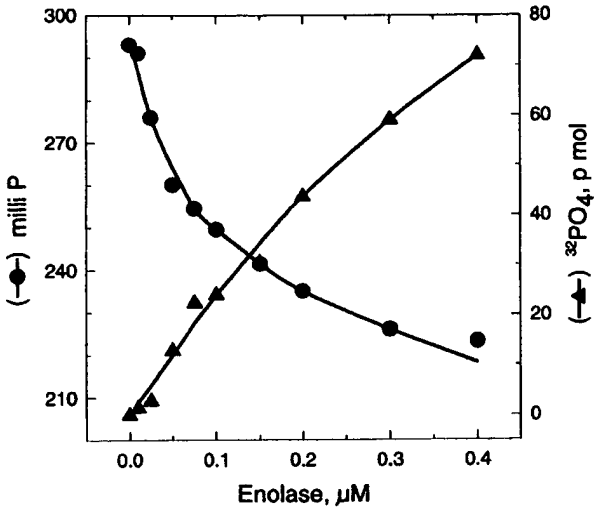

FIG. 6. Lck activity as a function of enolase concentration. Enolase phosphorylation increased with increasing enolase concentration and the FP signal decreased correspondingly. The competition of phosphorylated enolase with fl-phos-peptide suggests that the FP-competition immunoassay can be used with natural protein substrates in addition to peptide substrates. The solid lines are curves fitted with Poly.fit of Sigma Plot.

When enolase was used as substrate, $^{32}PO_4$ incorporation increased with increasing concentrations of enolase reaching 72 pmol at 0.4 μM enolase (Fig. 6). FP signal decreased with increasing amounts of enolase from 295 milli-P reaching about 230 milli-P at 0.4 μM enolase. With the peptide substrate, higher substrate concentrations were possible, allowing larger signals in both the FP and $^{32}PO_4$-transfer assays. Though the drop in the FP signal was not as dramatic as with the peptide substrate, the magnitude of the drop in the FP signal was inversely proportional to the $^{32}PO_4$ incorporated and was similar in magnitude to the drop in FP signal for a comparable incorporation of $^{32}PO_4$ with peptide substrate as shown in Fig. 5. Thus, with a protein substrate, both the FP-competition immunoassay and the $^{32}PO_4$-transfer assay showed similar dependence on substrate concentration, validating the usefulness of the FP assay for natural protein substrates.

Inhibition of Lck activity by inhibitors. To show that the FP-competition immunoassay will successfully detect inhibitors of Lck, inhibition by staurosporine, a potent nonspecific protein kinase inhibitor, and by PP, a specific Lck/Fyn inhibitor competitive with ATP (15), was evaluated at both 5 and 20 μM ATP. The inhibition curves were similar in both FP and $^{32}PO_4$-transfer assays (Fig. 7). The IC_{50} for staurosporine were 30 and 33 nM at 5 μM ATP and 110 and 95 nM at 20 μM ATP, in the FP and $^{32}PO_4$-transfer assays, respectively. IC_{50} for the Lck inhibitor PP were 0.070 and 0.075 μM at 5 μM ATP and 0.30 and 0.14 μM at 20 μM ATP, in the FP and $^{32}PO_4$-transfer assays, respectively. The inhibition obtained with staurosporine and PP using Lck-peptide substrate in this FP-competition immunoassay was within the expected sub-micromolar range (15). These IC_{50} values were very similar to the

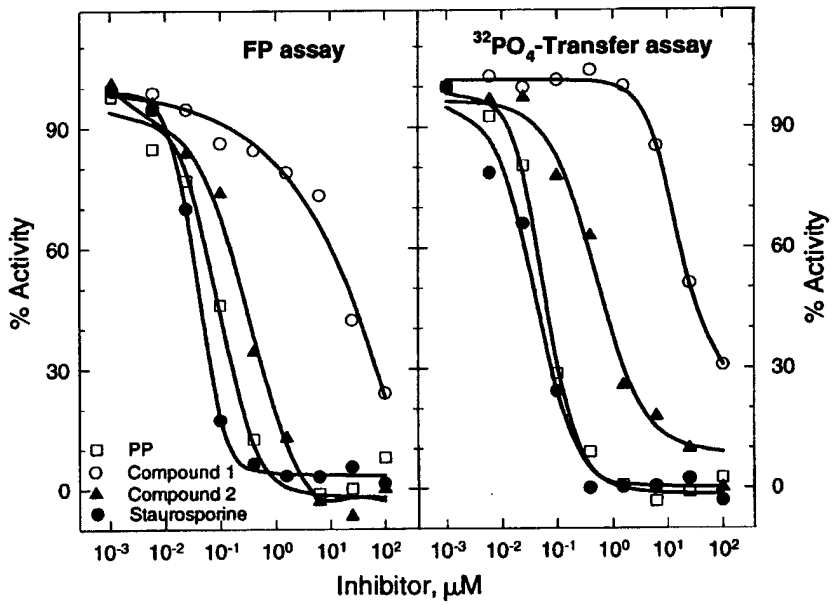

FIG. 7. Inhibition of Lck activity by staurosporine, PP, and other proprietary Lck inhibitors in the FP-competition immunoassay (left) and $^{32}PO_4$-transfer assay (right). The inhibition of Lck activity by increasing concentrations of inhibitors was measured at 20 μM Lck-peptide, 100 ng Lck, and 5 μM ATP and also with 20 μM ATP (data not shown). Inhibition was similar in both FP and $^{32}PO_4$-transfer assays. The solid-line curves are fitted with Logistic.fit.

TABLE 1

Comparison of IC_{50}s for Inhibitors in Lck Assay by FP and $^{32}PO_4$-Transfer Methods

	FP-method 1 (nM)				FP-competition immunoassay (nm)			
	ATP, 10 μM		ATP, 25 μM		ATP, 5 μM		ATP, 20 μM	
	FP	PO_4	FP	PO_4	FP	PO_4	FP	PO_4
Staurosporine	33	41	120	140	30	33	110	95
PP	210	70	400	225	70	75	300	140

Note. IC_{50}, the concentration of inhibitor required for 50% inhibition of activity, was determined for staurosporine and PP by FP-direct method (FP-Method 1) and $^{32}PO_4$-transfer assays that were run in parallel. Because PP is a competitive inhibitor with respect to ATP, the effect of inhibitors at two concentrations of ATP, 10 and 25 μM, was determined and the IC_{50} values are given in nanomolar concentrations. The IC_{50} values for staurosporine and PP were also determined by FP-competition immunoassay and concurrently run $^{32}PO_4$-transfer assays at 5 and 20 μM ATP. The IC_{50} values are averages of two independent determinations for fluorescence polarization assay (FP) and $^{32}PO_4$-transfer assay (PO_4).

values obtained in a FP-direct assay (Table 1). Similar IC_{50} values (Fig. 7 and Table 1) and enzyme and substrate saturation curves (Figs. 3–6) are found in both the $^{32}PO_4$-transfer assay and the FP-competition immunoassay, showing that our new procedure is a reliable alternative to the $^{32}PO_4$-transfer assay.

The FP-Lck assay we previously described is a very simple one-step method and is an excellent alternative nonradioactive method, but as discussed in the Introduction, it has some drawbacks. These problems are eliminated in the FP-competition immunoassay described in this paper. In the new approach the substrate can be a peptide, protein domain fused to GST, or a physiological relevant protein substrate. The phosphorylated substrate formed in this kinase reaction competes with a fluorescent phosphopeptide (used as a tracer) for phosphotyrosine antibody similar to the established immunoassays. This FP-PTK assay is very simple, nonradioactive, and highly sensitive and does not involve separation of substrate and product. A variation of this method would also be suitable for the assay of phosphatases. The simplicity and speed of this method make it ideal for high-throughput screening of compounds for tyrosine kinase inhibitors.

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